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13. ABSTRACT (Maximum 200 words)

Plate count methods are used to determine the efficacy of decontaminants against pathogenic bacteria. However, they are costly, labor intensive and time-consuming. With recent concerns of biowarfare, a simple and rapid means for characterizing decontaminant formulations and monitoring clean up efforts are greatly needed. Luna has developed several fluorescence-based assays to monitor the viability of microbes such as *E. coli*, *S. aureus* and *B. globigii,* the anthrax simulant. Dose-response and time-course lethalities to decontaminants can be determined within 1-2 hours. During this special contract, Luna Innovations adapted the fluorescent viability assays to monitor the decontamination of bacterial spores. With the addition of an acceleration step, the resazurin viability assay monitored the lethal effects of decontaminants on spores of *B. globigii* with good correlation to decreases in plate counts (spores/ml). Spores were recovered from glass surfaces decontaminated with DF-200 and processed using a novel syringe/filter sampling format. By combining the acceleration step with the syringe/filter formats, as few as 5-50 spores were detected from swabbed surfacesin 8-10 hours. This rapid, fluorescence-based viability assay has been formatted for "high throughput" use with a fluorescent microplate reader and provides a rapid alternative to extensive primary plate count screening.

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(Continuation Sheet)

Tables of Contents

1. Summary of Work Plan

The proposed general work plan for the project, as depicted in the original proposal, consists of three major technical objectives; 1) develop rapid spore sampling and washing methods, 2) optimize the acceleration of bacterial spores and 3) refine the fluorescent viability formats for assaying accelerated spores. The proposed studies can be delineated according to the tasks listed in Table 1 and the progress on each is shown.

Table 1. Summary of tasks for technical objectives

Task #1	Grow, maintain and store spores of *Bacillus globigii*	Complete
Task #2	Model surfaces contaminated with bacterial spores	Complete
Task #3	Optimize the collection and enumeration of spores from treated surfaces	Complete
Task #4	Optimize the acceleration of spores and growth of viable cells	Complete
Task #5	Integrate the accelerant and assay reagents into syringe/filtration assembly	Complete
Task #6	Demonstrate monitoring of decontamination of spore-coated surfaces	Complete
Task #7	Format the assays for 96-well microplate throughput.	Complete

To date, no patents, patent disclosures, manuscripts or other published materials have resulted from the project. The scientific progress and accomplishment for the project are summarized in the following section.

2. Progress Report

Background

The recently completed DoD Phase I project "Viability Assay for Monitoring Decontamination of Pathogenic Bacteria" (DAAD19-02-C-0074), for which Luna is currently receiving Phase II funding, demonstrated the ability of fluorescence-based viability assays to augment the costly and time-consuming plate count methods for determining the

lethality of decontaminants on pathogenic bacteria. The fluorescent assays are based on measuring metabolic "markers" in cultures such as acid-production (pH change) and reduction and oxidation potential. Figure 1 shows the responses of the three tester strains, *E. coli* O157:H7, *S. aureus* Cohen, and *B. subtilus* var *globigii* (a.k.a., *B. globigii*) in the three fluorescent assays using SNARF-4F (pH), resazurin (reduction) and dihydrodichloro-fluorescein (DCF, oxidation) as the indicator fluorophores.

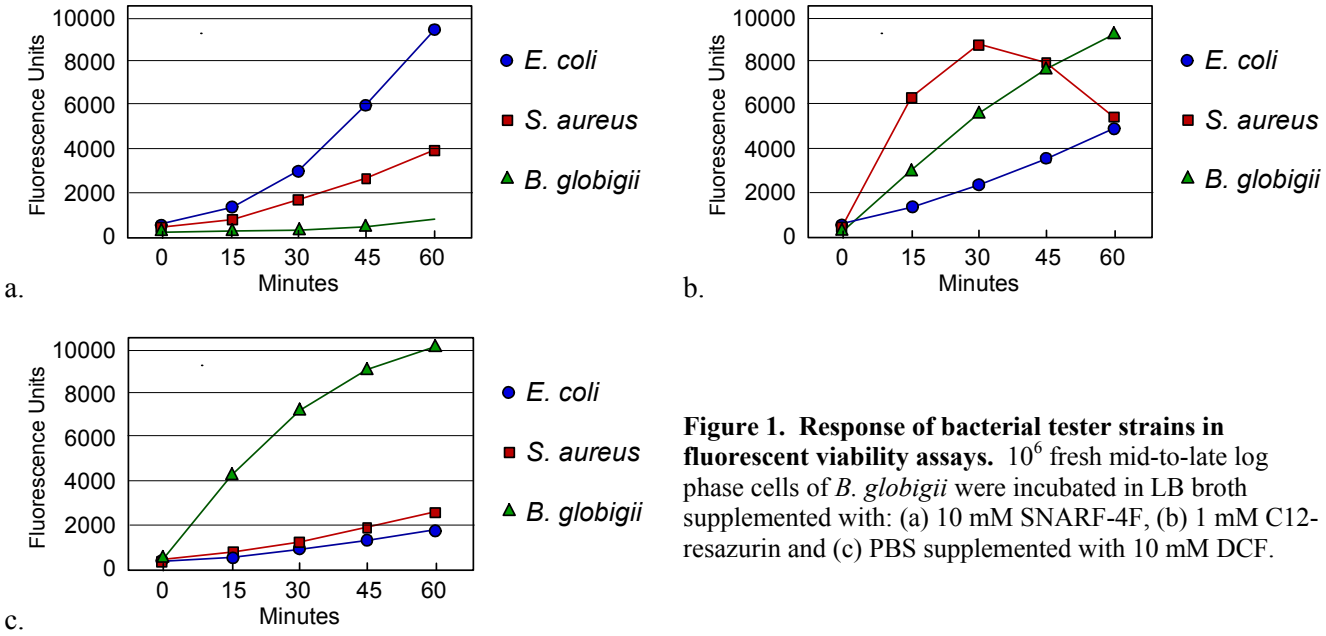


Figure 1. Response of bacterial tester strains in fluorescent viability assays. 10^6 fresh mid-to-late log phase cells of *B. globigii* were incubated in LB broth supplemented with: (a) 10 mM SNARF-4F, (b) 1 mM C12-resazurin and (c) PBS supplemented with 10 mM DCF.

In the final month of the aforementioned Phase I, the ability of the fluorescent assays to monitor the efficacy of the decontaminant DF-200 against vegetative cells and germinated and outgrown "accelerated" spores of *B. globigii* was demonstrated (Figure 2). DF-200 is a triple component quat and peroxygen-based decontaminant on which the final studies during the Phase I program focused. These preliminary data became the focus of the current DoD project presented herein, "Monitoring the decontamination of bacterial spores using fluorescent viability assays" (DAAD19-03-C-0068).

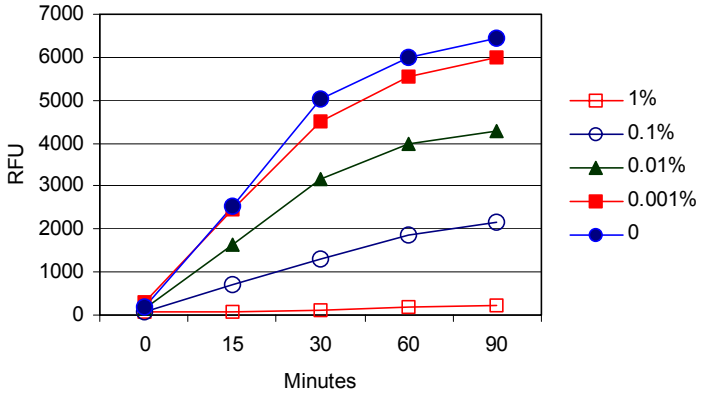


Figure 2. Responses of vegtetative *B. globigii* to DF-200. The three components of DF-200 were mixed and used within 30 minutes. Cell suspensions (~10^6 CFU/ml) were incubated with 1-0.001% DF-200 for 30 min at 37ºC. The cells then were washed and assayed in the DCF assay for 90 minutes.

The last studies in Phase I were designed to demonstrate that the efficacy of decontaminants against bacterial spores also could be monitored using the viability assays with an additional acceleration step in the protocol. Spores of *B. globigii* can be easily and rapidly germinated and outgrown (a.k.a., accelerated) in Luria-Bertani (LB) broth supplemented with alanine and inosine. By accelerating spore preparations before and after treatment with a decontaminant and then testing the resulting outgrown cultures for overall viability, one can determine the lethality of the decontaminant. A dose-response of *B. globigii* spores treated with DF-200 is shown in Figure 3.

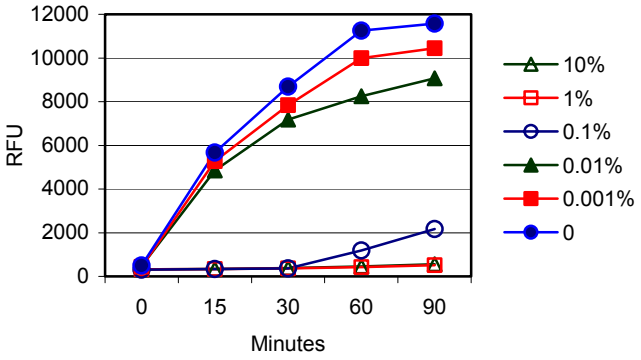


Figure 3. Response of *B. globigii* spores to DF-200 in the DCF assay. Fresh spore suspensions (`10^6) from LB stock plates were incubated with DF-200 for 30 min at 37ºC. Spores were then washed, germinated and outgrown (accelerated) in LB broth containing 10 mM L-alanine/1mM inosine for 5 hours at 37˚C prior to assaying in the DCF assay.

For the current spore decontamination project, we proposed to further optimize the spore acceleration process and adapt the DCF and/or resazurin fluorescent viability assays for monitoring decontamination of spore-treated surfaces.

2.1 Growth, maintenance and storage of *Bacillus globigii* spores

B. globigii is a common reference tester strain for examining the sporicidal effects of antiseptics and disinfectants. It is also used in our laboratory as a simulant for *Bacillus anthracis* in the development of fiber-optic and fluorescence-based biosensors for detection of biowarfare agents. *B. globigii* was grown at 37˚C in Luria-Bertani (LB) broth and maintained on LB agar slants at 4˚C. Frozen stocks were prepared by adding 10% sterile glycerol to late-log LB cultures and storing them at –80˚C. Fresh spores were prepared by spreading 0.1 ml of a fresh LB culture onto a nutrient sporulation agar medium (0.3% yeast extract, 0.3% tryptone and 0.001% $MnCl_2$) and allowing growth to confluence at 37˚C. Spores then were harvested by scraping them off the agar surface using a sterile spatula, resuspending them in PBS and dispersing them at 4˚C in a sonicating water bath. The spore stocks were stored at –80• until used.

Viability of the standard spore stocks was confirmed by standard serial dilution and enumeration by plate counts. Prior to plating, spore preparations were heat shocked at 70˚C for 10 min. One-tenth ml of ten-fold serial dilutions of the heat shocked spore stocks were spread using disposable spreading loops onto duplicate LB plates. The plates were incubated at 37˚C for 18-24 hours, the CFU/ml of the spore stocks were determined and 1 ml aliquots were stored at –80˚C until used. The frozen spore stocks were ~5 x 10^8 spores/ml and their viability was not affected by short or long term freezing at –80˚C.

2.2 Model surfaces contaminated with bacterial spores

To model the contamination of surfaces with bacterial spores in such a way that reflect a release during biowarfare or bioterrorism, Luna adapted the methods currently used by the contractor. Because of the difficulty in accurately enumerating the number of spores applied to a surface by spraying, sterile glass slides (4 x 5 cm) were coated with 1 ml of known PBS dilutions of standard spores and dried overnight at room temperature. The spores were removed from the air dried plates using Dacron swabs moistened with phosphate buffered saline (PBS) and the spores were recovered from the swabs by suspension in 1 ml PBS.

Enumeration of the viable spores was performed using a standard plate count technique whereby duplicate 0.1 ml aliquots of the diluted swab suspensions were spread onto the entire surface of nutrient agar plates and incubated at 37˚C for 18-24 hours. To estimate the recovery efficiency, plate count results from the swabbed glass slides were compared with the count determined by enumeration of the original dilutions. In addition, we directly enumerated the spores dried on the slide by pressing the slide face down onto the surface of an agar plate for 30 minutes, aseptically removing the glass slide and incubating the agar at 37˚C for 18-24 hours.

2.3 Optimizing the collection and enumeration of spores from treated surfaces

To determine the effect of the suspension buffer on the application of the spore to the glass slides we prepared suspensions of spores in PBS, 10% PBS or sterile water and dried each onto the surface of the slides. With the PBS, a heavy precipitate of salts was evident after drying which impeded the swabbing step. With the 10% PBS and water

suspensions, swabbing was more facile and 10% PBS was chosen as the standard diluent in that it left a slight residue with which we could assure complete swabbing of the spore-coated region of the slide. The results from the plate counts on the spore-coated slides are shown in Table 2.

Table 2. Plate counts and swab recovery of *B. globigii* spores

	Control Tube	Plate Swab	Contact Plate
Dilution			
1.00E-05	4436	3833	TNTC
1.00E-06	435	401	427
1.00E-07	40	33	43
1.00E-08	<10	<10	6
Control	0	1	0

The plate counts from the swabbed slides were not affected by any of the suspension media and the recovery of spores from glass slides using Dacron swabs was generally 80-90%.

2.4 Optimizing the acceleration of spores and growth of viable cells

2.4.1 Acceleration of spores

The ability of spores to germinate efficiently with chemical rather than heat activation has been demonstrated with several species of *Bacillus*. To confirm that this was the case for *B. globigii*, and to show that a separate activation step was not necessary in our spore acceleration model, we compared the growth of spores treated in a variety of ways prior to accelerating in LB accelerant (LB+alanine+inosine) for 2 hours and enumerating on LB agar (Figure 4).

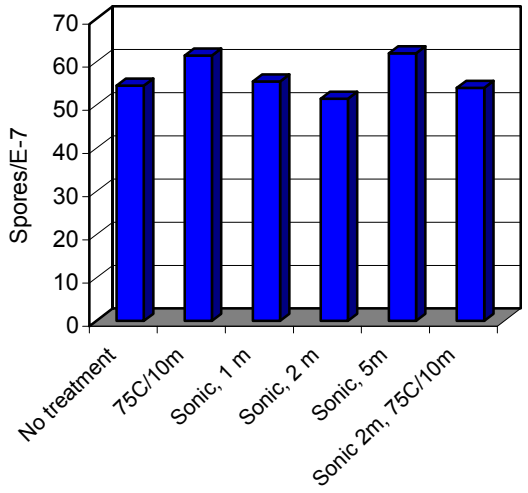


Figure 4. Effects of conventional spore activation methods on spore acceleration in LB/alanine/ inosine. Spores were treated and incubated in LB accelerant at 37˚C for 2 hours prior to plating on LB agar.

The spore counts on accelerated preparations of non-activated and heat-shocked spores were essentially the same indicating that an initial activation step is not required for efficient germination and outgrowth into healthy viable cells. Sonication did not affect the counts indicating that the spores were relatively free in suspension and not aggregated by hydrophobic interaction.

2.4.2 Growth of accelerated spores

To determine the time-course of spore acceleration, spores were diluted in LB accelerant broth (LB+10 mM alanine and 1 mM inosine; LBA) and the dilutions were incubated at 37˚C for 8 hours. Figure 5 shows the growth curves for each dilution as measured by turbidity at 600 nm.

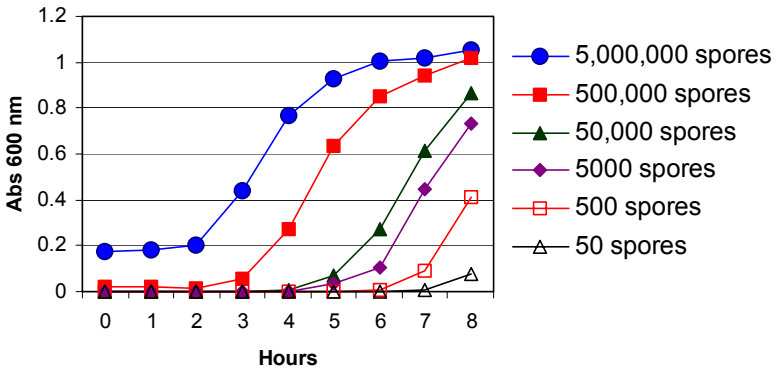


Figure 5. Growth curves of *B. globigii* from spores in LB/accelerant (LBA) broth. A frozen stock of *B.g.* spores (~5x10^8/ml) was diluted in LBA using serial 10-fold dilutions. 1 ml of each dilution was incubated in 13x100 mm culture tubes at 37ºC with shaking at 240 RPM.

Figure 6 shows the spore and post-acceleration viable cell counts as determined by plate count enumeration on LB agar. The germination/outgrowth phase occurs within the first 2 hours after which the growth proceeds exponentially with doubling times of 25-30 minutes between hours three and eight.

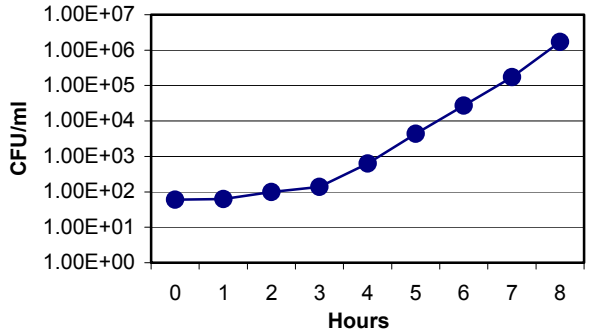


Figure 6. Growth of spores diluted in LBA. Spores were diluted to 10^{-7} in LBA (~50 spores/ml) and incubated at 37ºC as describe in Figure 5. Plate counts on LB agar were performed each hour.

To demonstrate the inaccuracies that can arise by direct enumeration of outgrown spores using plate count methods, sonicated spore preparations were compared to non-sonicated spores following 3 hours of acceleration in three different culture broths (Figure 7). In all accelerated cultures, sonication indicated that considerable aggregation of viable cells occurred. This would result in a significant underestimation of cell numbers during decontamination studies and must be taken into consideration when doing these types of experiments. Conversely, sonication had no effect on the responses in the viability assays which measure total bacterial numbers and not CFU/ml.

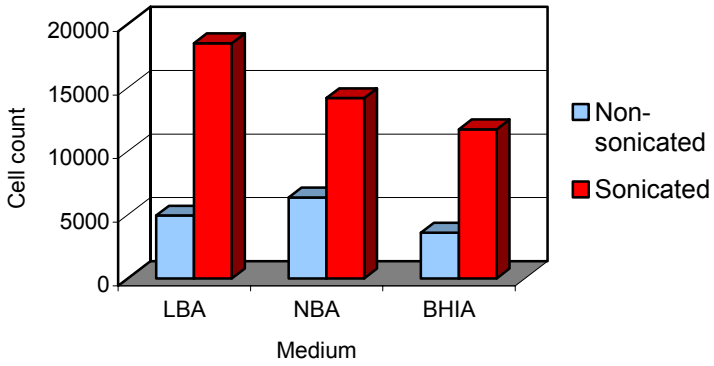


Figure 7. Effect of sonication on enumeration of accelerated spores. Spores were diluted to 10^{-7} in accelerants based on LB broth (LBA), nutrient broth (NBA) and brain heart infusion broth (BHIA) and incubated at 37ºC for 3 hours. Aliquots were then placed in a sonic bath at 37˚C for 2 minutes prior to plating.

LBA was chosen as the standard accelerant for all subsequent studies because it appears to best promote germination and outgrowth and is the least complex of the media, thus causing the lowest background and assay interference.

2.5 Integrate the accelerant and reagents into the syringe/filtration format

During the development of the syringe/filtration assay design, several parameters of the format had to be investigated and optimized. These include, i) the proper choice of fluorophores, ii) the proper derivative of the fluorophores, iii) the bacterial growth characteristics within the syringe and iv) the choice of membrane filter. The following section discusses these and how they all integrate into the final assay format.

2.5.1 Viability assays - DCF vs Resazurin

During previous preliminary studies on the viability assays developed at Luna, the DCF assay was modeled with vegetative *B. globigii* because it was the only tester strain that had significant oxidative potential for the fluorogenic substrate. However, *B. globigii* also exhibits a strong response in the resazurin assay (Figure 1) and thus studies comparing DCF to resazurin were performed early in this project to determine which would work best with accelerated spore preparations. Spores were accelerated into mid-log phase in LBA for 5 hours and serial 10-fold dilutions were tested in the standard DCF and resazurin assays for up to 3 hours. (Figure 8).

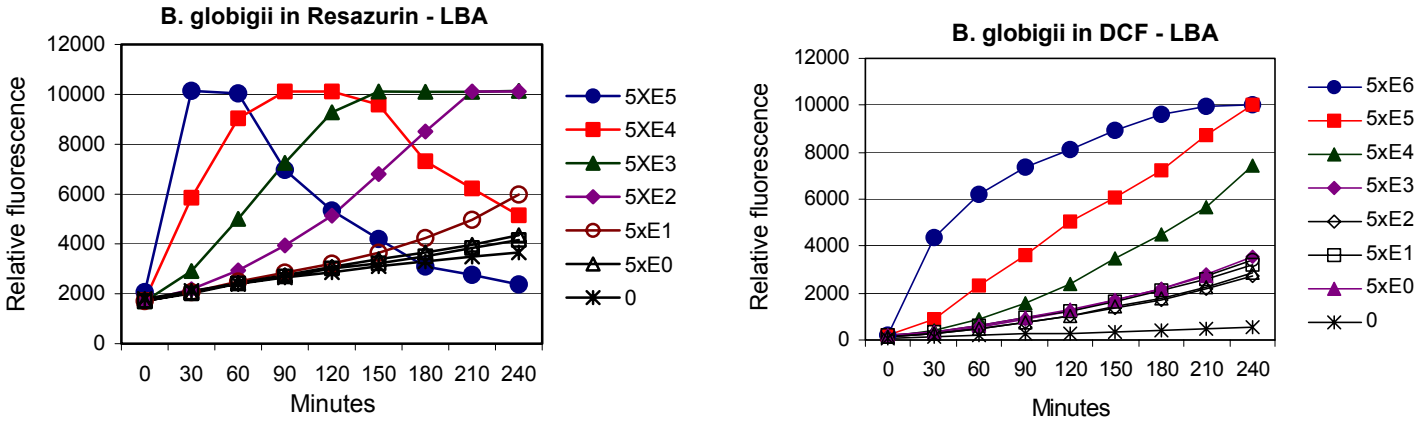


Figure 8. Dose-response comparison of *B. globigii* in the resazurin and DCF assays in LB/accelerant broth. Serial 10-fold dilutions of a frozen spore stock (10^{-8} spores/ml) were made in the LB/accelerant (LBA) and grown for 5 hours at 37˚C. The germinated and outgrown LBA cultures were then tested in the DCF and resazurin assays.

The sensitivities of the two assay formats are shown in Figure 9. Whereas the DCF assay can detect between 10^4 and 10^5 cell/ml, the resazurin assay can detect between 10^3 and 10^4 cells/ml. In addition to being more sensitive, with the resazurin assay medium components do not significantly affect the assay response. With DCF, all media caused strong oxidation background signals thus the assay must be performed on washed cells. With resazurin, the fluorescence-response can be measured directly in the LBA culture.

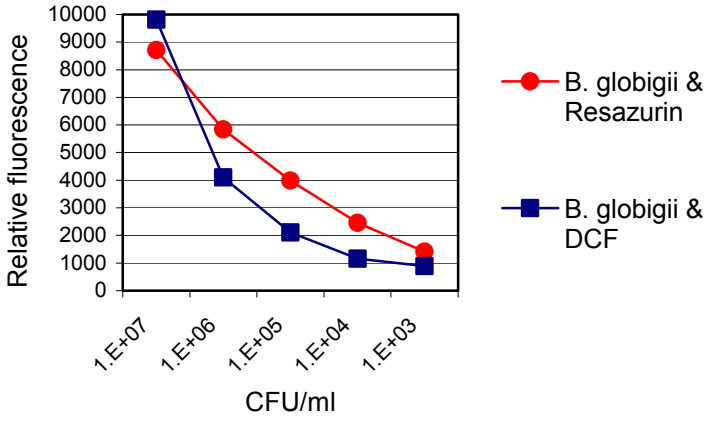


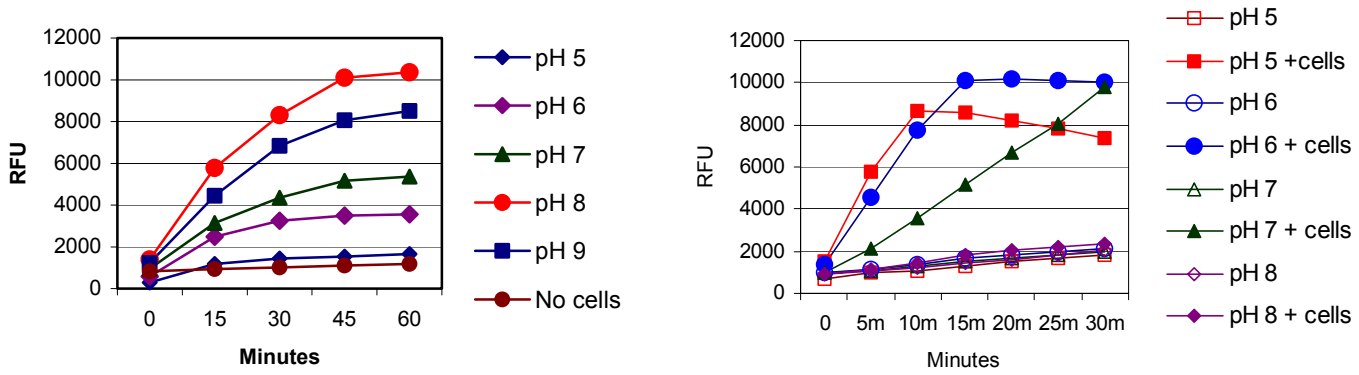
Figure 9. Dose-response of *B. globigii* in the DCF and resazurin assays. B. globigii was serially diluted 10-fold and assayed in the respective assay format for 1 hours at 37˚C.

The resazurin assay resulted in a more sensitive and more linear signal response with which the relationship of cell numbers and fluorescence units can be determined. In that the resazurin assay is a log more sensitive than the DCF assay, it was chosen as the primary assay format for subsequent spore studies.

2.5.2 C_{12}-resazurin vs sodium resazurin

Initial studies on the resazurin assay used a C_{12}-derivative of resazurin from Molecular Probes designed for more easy access by mammalian cells. It is quite expensive and because we were only measuring its reduction by bacteria in vitro, we tested whether or not the C_{12} form could be replaced with a less expensive, more freely soluble sodium salt form. To determined pH optima, LBA broths adjusted to different pH and supplemented with C_{12}-resazurin (1 uM) and sodium resazurin (2.5 µM) were inoculated with vegetative *B. globigii* (~10^6 CFU/ml) and incubated at 37˚C for 1

hour. Figure 10 shows the considerably different pH optima for each of the forms of resazurin. The sodium resazurin exhibited stronger, more rapid signal responses than the C_{12} form with a more biologically neutral pH optimum of ~6.



a. b.

Figure 10. Determination of the optimal pH for the C12 and sodium resazurin in the fluorescent viability assay.
a. C_{12} resazurin had a pH optimum at pH ~8, b. sodium resazurin had a pH optimum at pH ~6.

When all three tester strains were tested against the two forms of resazurin at their respective pH optima, the sodium form yielded greater signal responses in all cases (Figure 11). Thus, we used the sodium salt of resazurin for most subsequent spores studies.

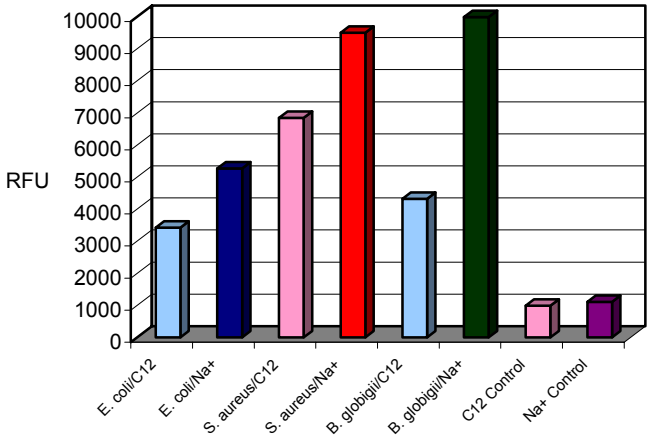


Figure 11. Comparison C12 and sodium resazurin in the fluorescent viability assay. The three standard tester strains were tested against each resazurin using the standard assay format (i.e., 10^6 cell/ml, 37˚C, 1 hour, 240 RPM)

When dilutions of spores were accelerated for 5 hours and tested in the viability assay using sodium resazurin (Figure 12), similar sensitivities as observed previously with C_{12}-resazurin were observed. Significant increases in fluorescence were observed in the preparations derived from as few as 50 spores.

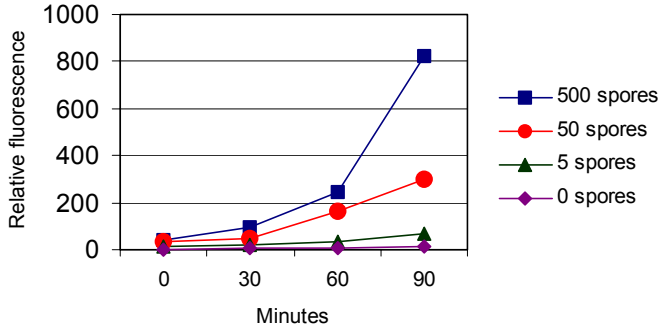


Figure 12. CFU-related responses of *B. globigii* in sodium resazurin. Spores were accelerated for 5 hours at 37˚C in LBA. Cells were diluted 1/10 in LB/resazurin reagent and monitored for fluorescence over time.

When dilutions of spores were coated onto glass plates, recovered with Dacron swabs, accelerated at 37˚C for 7 hours using prewarmed accelerant and assayed in prewarmed resazurin reaction mixture, significant increases in fluorescence were observed with as low as ~ 5 spores/glass slide. Based on these dose-related studies, in an 8-9 hour

period (one working day) as few as 5-50 spores can be detected (Figure 13). Thus, the lethality of a decontaminant on heavy spore loads should be able to be determined to this minimal level.

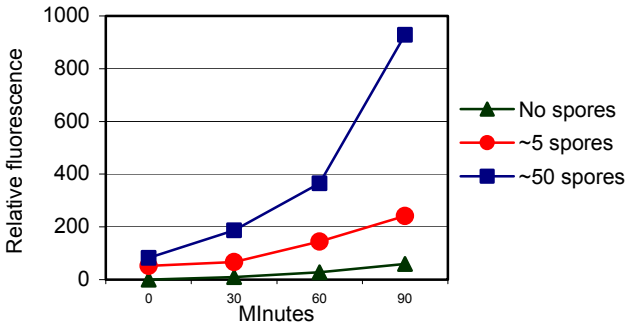


Figure 13. Responses of swabbed spores 7 hours after acceleration. Spore were incubated at 37C, shaking at 240 RPM for 7 hours. Background fluorescence was subtracted from experimental values.

2.5.3 Syringe/filter assay format

Following optimization and refinement of the separate components of the spore detection scheme, i.e., swabbing, recovering, accelerating and assaying, we integrated them into a simple syringe/filter assay format. The direct syringe/filter format involved recovering spores from swabs directly into LB/accelerant (LBA) within a 5 ml syringe attached to a plugged 0.45 micron membrane filter. The plunger then was placed onto the syringe, the assembly was inverted and the syringes were incubated at ~30-45˚ angle (to prevent leakage) at 37˚C for 7 hours at 240 RPM. This approach is diagramed in Figure 14.

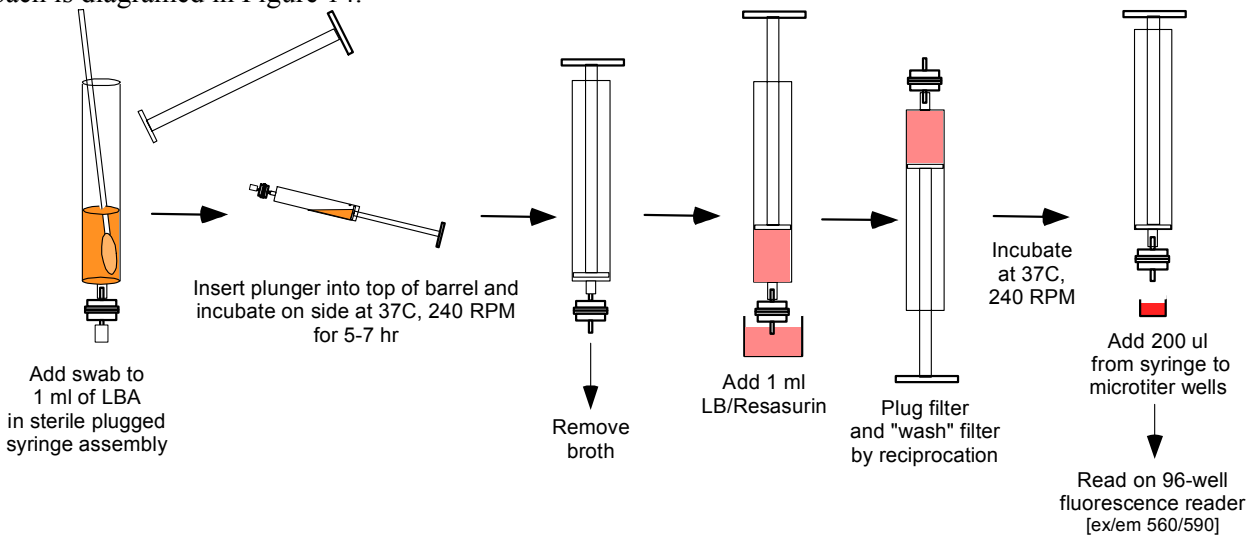


Figure 14. Schematic of syringe/filter assembly used for monitoring spore viability with the resazurin viability assay.

2.5.3.1 Growth characteristics

Prior to examining the potential of the syringe/filter format for rapid assessment of spore viability, several aspects of the format were investigated individually. These included, i) confirming that the spores accelerated properly in the syringe, ii) assuring that the resazurin was not bound to the filter during cell processing, and iii) determining which filter type most easily allowed for extrusion of cells and uptake of the LBA and resazurin reagent.

To confirm that the spores accelerated properly in the syringe, dilutions of spores containing ~50 spores and ~5 spores were grown in LB with and without alanine/inosine and in LBA in 5 ml syringes (Figure 15). After 7 hours incubation, the cultures were enumerated by the plate count method and the CFU/ml were compared. Both spore suspensions grew well in the accelerated cultures and in both cases the syringe culture grew slightly better than in the glass tubes. No problems with growth of the cells in syringes were encountered and the culture derived from the 50 spores grew to cell densities easily monitored by resazurin.

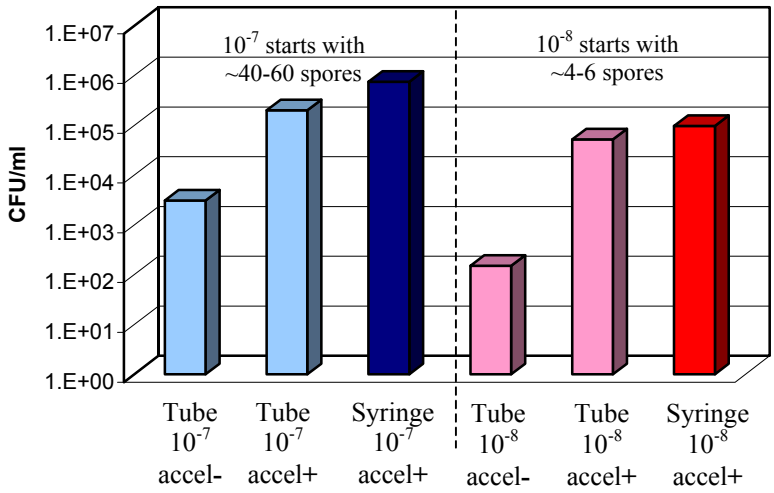


Figure 15. Effects of accelerant conditions on outgrowth of *B. globigii* spores – tube cultures vs syringe cultures. 10^{-7} and 10^{-8} dilutions of spore stocks in LB broth, containing ~50 and ~5 spores, respectively, were incubated without (accel-) or with (accel+) the alanine/inosine accelerant for 7 hours at 37˚C and enumerated by plate count on LB agar.

2.5.3.2 Membrane filters

During preliminary studies with the C_{12}-resazurin in the syringe/filter assembly, considerable binding of the fluorophore to cellulose acetate membrane filters was observed. In addition, the C_{12} form adsorbed to the inside of the syringe barrel thus rendering the assay incapable of monitoring cell viability. When we adapted the sodium resazurin to the protocol, the syringe adsorption was no longer a problem but significant amounts of the sodium salt still bound to the membrane filter. Consequently, we investigated several filter types for a non-binding membrane that could be used in the syringe/filter assay format. Using absorbance at 600 nm as an indicator of relative resazurin concentrations, two ml of LBA supplemented with 2.5 uM sodium resazurin was drawn up into the syringe/filter assembly and the absorbances of the reagents were determined (Table 3). At the same time, the relative ease with which the reagents could be blown completely out of the syringe (causing potential "airlocks") and drawn back up was determined.

Table 3. Comparison of resazurin binding and flow characteristics of various filter types.

Filter type (0.45 µm)	Abs (600)	Filter Binding	Relative ease of reagent flow
No filter	0.54	n/a	n/a
Cellulose acetate	0.12	strong	moderate resistance to flow out; poor flow in
Polypropylene	0.42	partial	slight resistance to flow out; poor flow in
Polysulfone	0.52	none	slight resistance to flow out; moderate flow in
Nylon	0.51	none	slight resistance to flow out; moderate flow in
Microcrystaline glass	0.53	none	free flow out; minimal resistance flow in

The best filter for use with the assay format was the microcrystaline glass membrane that showed very little resistance to drawing up sample. Although the polysulfone and nylon filter did not adsorb resazurin, they were hard to fill by drawing up reagent through the filter and required continual tapping to break the air/liquid surface tension within the membrane.

2.5.3.3 Integrated syringe/filter resazurin assay format

Following evaluation and optimization of the individual components syringe/filter format, we performed various experiments to appraise several iterations of the complete assay design. First, we tested diluted mid-log phase cells in a simplified format whereby the cells were washed by filtration, the resazurin reagent was drawn into the syringe resuspending the cells and the fluorogenic responses were measured (Figure 16). As was observed in previous studies

with cell dilutions in the culture tube format, significant responses were observed in the syringe/filter format at cell concentrations of 5000/ml and above.

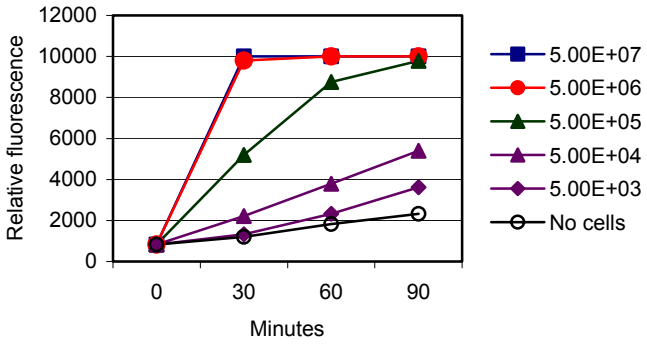


Figure 16. Dose response of viable cells of *B. globigii* in the syringe/filter format. Fresh mid-log phase cells of *B globigii* were diluted in PBS and 1 ml was added to syringe/filter assemblies. The PBS was removed by filtration, the LB resazurin reagent was drawn up into the syringe assemblies and the reaction mixtures were further incubated at 37˚C for 90 minutes.

Next, we tested diluted spores in a similar format whereby after acceleration, the accelerant was removed by filtration and processed as above for the resazurin viability reaction (Figure 17). As observed in the culture tube format, after ~7 hours acceleration the cultures resulting from 50 spores gave significant responses and the responses of the culture derived from five spores were close to significant.

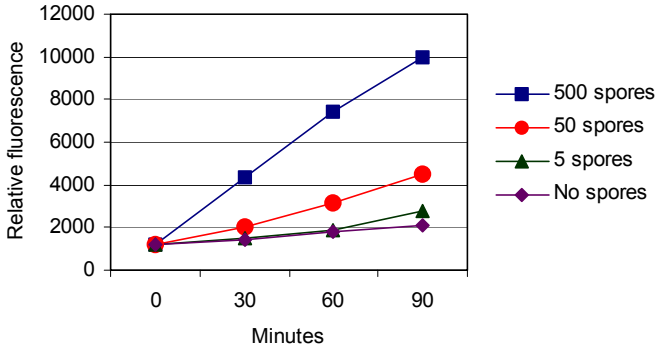


Figure 17. Dose response of accelerated spores of *B. globigii* in the syringe/filter format. Dilutions of spore stocks were accelerated in 1 ml LBA in the syringe/filter assembly at 37˚C for 7 hours. The accelerant was removed by filtration, the LB resazurin reagent was drawn up into the syringe assembly and the reaction mixtures were further incubated at 37˚C for 90 minutes as depicted in Figure 14.

The final iteration of the syringe/filter assay format involved the more complex approach needed when testing decontaminants that interfere with the viability assay. It involves several steps including, i) recovering spores from swabs in buffer directly in the syringe, ii) washing the spores (to remove the decontaminant), iii) drawing LBA into the syringe for 7 hours acceleration of the spores, iv) removing the accelerant by filtration and drawing resazurin reagent into the syringe for the viability reaction and measurement (Figure 18).

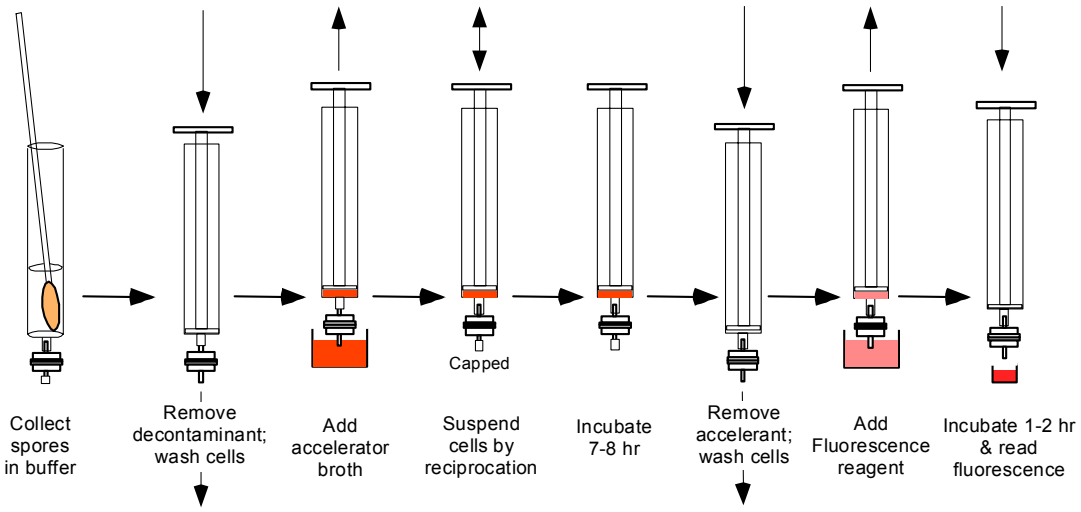


Figure 18. Syringe format for monitoring spore lethality on contaminated surfaces. A more complex iteration of the syringe/filter format is needed for situations in which the decontaminant, the accelerant, or both, interfere with the viability assay.

When using a viability assay like the resazurin assay in which neither the decontaminant nor the accelerant interferes with the response, the syringe format is not necessary. Swabbed spores can be directly suspended in accelerant in sterile culture tubes, incubated for 7 hours and the resazurin can be added directly to the tubes for viability measurements. Thus, depending on the situation, there are several viability assay formats that can be used to monitor the efficacy of decontaminants against bacterial spores on contaminated surfaces, as well as in laboratory screening of developmental formulations (Table 4).

Table 4. Comparative viability assay formats for monitoring bacterial spores

Interference	Resazurin	Resazurin Format*	DCF	DCF Format
Decontaminant	none/low	direct tube/simple syringe	high	complex syringe
Accelerant	none	direct tube	high	complex syringe
Decontaminant & accelerant	low/none	simple syringe	high	complex syringe

*Simple syringe, Figure 14; Complex syringe, Figure 18.

2.6 Monitoring decontamination of spore coated surfaces

Following the integration of four major components of the syringe/filter approach to monitoring decontamination of spores (i.e., swabbing, washing, accelerating and assaying), we performed comparative studies to determine the correlation between cell lethality and the responses of treated spores in the resazurin viability assay.

2.6.1 Plate count studies

Glass slides (2"x3") were cleaned with alcohol, spread with 1 ml of ~10^6/ml spores diluted in 5% PBS, pH 7.2 and dried for 4-6 hours. A set of duplicate plates were sprayed with ~0.1 ml DF200 (as measured by the increase in the weight of the slide) and dried overnight at room temperature. All slides then were swabbed thoroughly using vertical, horizontal and diagonal coverage with PBS-moistened Dacron swabs and recovered in 1 ml PBS. The recovered spores then were wash 5 times with 1 ml PBS and 1 ml of accelerant was drawn into the syringe resuspending the cells. The syringes were incubated upright at 37˚C for 7 and 9 hours. Positive controls included diluted spores directly processed in the syringe format with no decontaminant or swabbing. Figure 19 shows the results of plates swabbed from control slides and DF200-treated slides. No colonies were seen from any slide treated with DF200, regardless of how it was processed.

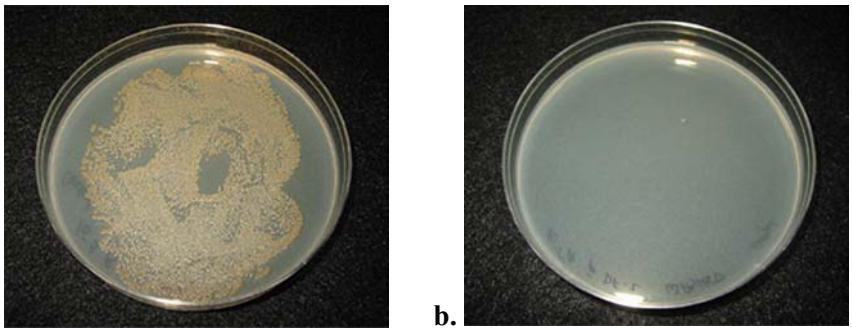


Figure 19. Swabs of glass plates coated with 10^6 spores. One ml (10^6 spore/ml PBS) was spread and dried onto a 2x3" glass slide. a. swabbed plate control after drying at room temperature overnight., b. swabbed plate after spraying with ~0.1 ml DF-200 and drying at room temperature overnight.

Determination of spore recovery and enumeration of controls and DF200-treated spores were performed using standard plate count methods. One ml of diluted spores (~10^6) was dried on duplicate glass slides, sprayed with 0.1 ml DF200, dried overnight and swabbed, syringe washed and accelerated for 7 and 9 hours. Accelerated cells were enumerated on LB agar plates at 37˚C for 18 hours (Table 5).

Table 5. Swab spore counts from slides decontaminated with DF-200.

Swabbed Slide	Counts–0 hours*	Counts–7 hours	Count-9 hours
Control - tube	1.73 x 10^6	1.98 x 10^8	3.22 x 10^8
Control - syringe	1.55 x 10^6	1.41 x 10^8	2.86 x 10^8
DF-200 - syringe	<10	<10	<10

The recovery of spores from the glass slide was ~85%-90%. During the 7 and 9 hours acceleration periods the viable counts of the controls increased greater than 2 logs to stationary growth phase. However, no viable cells were recovered from the DF-200 plates and duplicate contact plates pressed onto the surfaces of the LB agar showed no viable spores remaining on the DF-200 treated surfaces (as similarly shown in Figure 19).

2.6.2 Resazurin studies

As a control for DF-200 treatment of slide coated spores, untreated spores (5 to 50,000 spores per ml) were dried onto glass slides, recovered directly in 1 ml LBA using Dacron swabs and accelerated at 37˚C for 7 and 9 hours at 240 RPM. After 7 hours, ~50 accelerated spores gave significant responses; after 9 hours acceleration of ~5-10 spores resulted in significant resazurin responses (Figure 20).

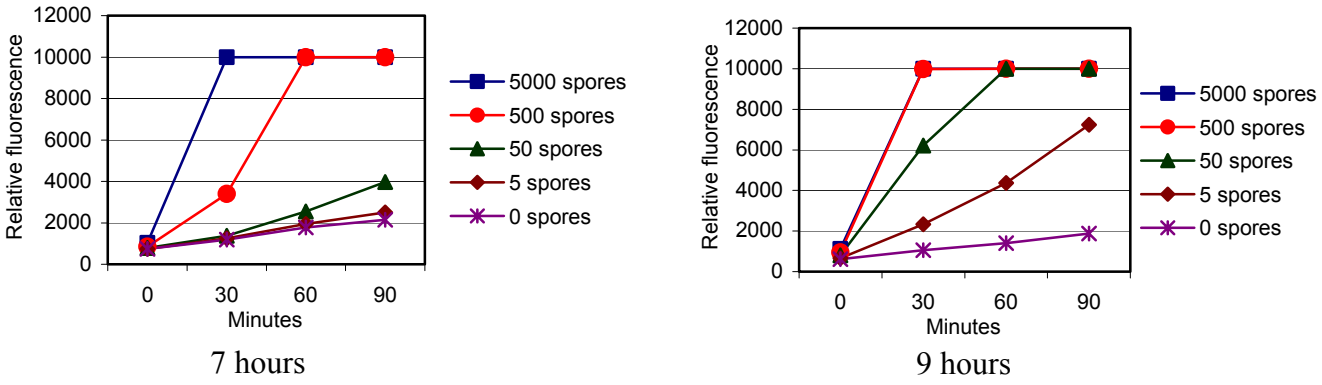


Figure 20. Control responses of accelerated spores in the resazurin viability assay. Known amounts of spores were recovered from glass slides and processed using the five ml syringe/filter acceleration format of the resazurin assay.

As shown in Figure 20, with a 7-9 hours acceleration period 5-50 spores can be detected using the multistep approached described above. As shown in previous dose-response studies, 10^4 to 10^5 viable cell are required for significant responses and this cell number can be attained during the 5-6 hours of acceleration following germination in which the doubling times are 25-30 minutes. Theoretically starting with one spore, after accelerating into a vegetative population as described above (i.e., 2-3 hours germination and outgrowth and 6-7 hours of 25-30 min doubling), the cell count should be 1-2 x 10^4 and in the detectable range of the assay.

The final feasibility studies involved the syringe/filter assay format to actually monitor the decontamination of spore-coated surfaces using DF200. Glass slides were coated with spores of *B. globigii* and treated with DF-200 as described above. Swabbed spores were washed 5x with 1 ml PBS, accelerated in 1 ml LBA for 7 and 9 hours, washed free of accelerant and tested in the resazurin assay as shown in Figure 21. Glass microfiber membrane filters (13 mm, 0.45 µm) gave best results. The spores from the untreated slides accelerated into heavily turbid cultures and gave maximum signals in the resazurin assay within 30 min. The DF-200 treated slides yielded no colonies and no differences were observed between 7 and 9 hours acceleration.

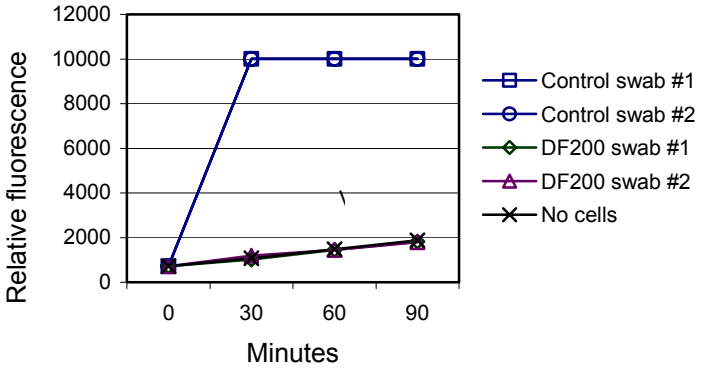


Figure 21. Responses of DF-200 treated spores swabbed and processed using the syringe/ filter assembly. Strong activity was observed in the resazurin assay with the untreated swabbed control spores and no activity was evident in the treated slides.

2.6 Microplate viability assays

In addition to using the microplate reader for rapid acquisition of experimental data from the tube and syringe/filter viability formats, it can be used to perform online, high-throughput accelerant and screening studies using fluorescent viability assays. Figure 22 shows absorbance curves automatically generated during the acceleration of dilutions of *B. globigii* spores. Using the Tecan GENios, heat-controlled, shaking fluorescent microplate reader, we generated a matrix of 200 µl minicultures derived from diluted spores incubated at 37˚C over 8 hours (Figure 22). The growth rates were similar to those obtained during previous growth studies using tube cultures and the microcuvette-based Beckman VersaFluor fluorometer.

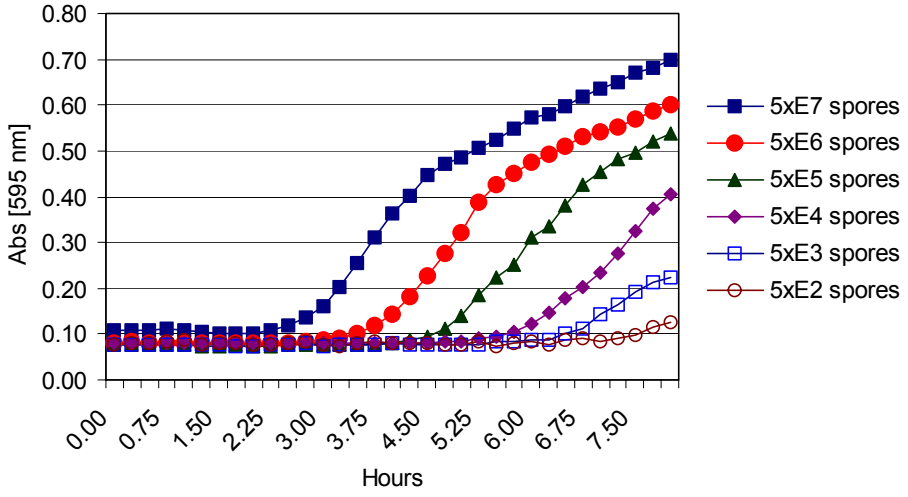


Figure 22. Growth curve from *B. globigii* spores using an automatic fluorescent microplate reader. Spores were diluted and accelerated at 37˚C, 120 RPM in the TECAN GENios reader for 8 hours. Growth was monitored by turbidity at an absorbance of 595 nm. Legend (right) delineates the number of spore inoculated into microplate wells.

To demonstrate the power of the rapid microplate format to test multiple and redundant samples and determine coefficients of variation, we examined the fluorescent responses of accelerated spores in a test matrix whereby quadruplicate samples were tested using two-fold dilutions prepared directly in the microplate wells. Accelerated spores were washed two times with PBS and 200 µl of each dilution were added to each of the 12 first wells. Then, 100 µl aliquots were transferred serially into 100 µl of the DCF reaction mixture through eight adjacent rows of wells resulting in a final dilution of 1/256. The reaction mixtures were incubated at 37˚C for 1 hour and fluorescence was read at ex465/em535 (Figure 23).

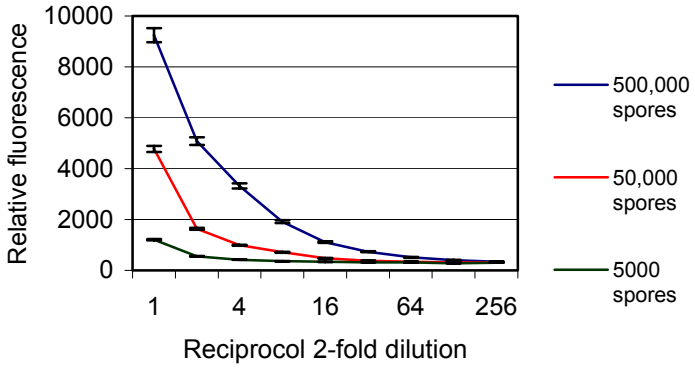
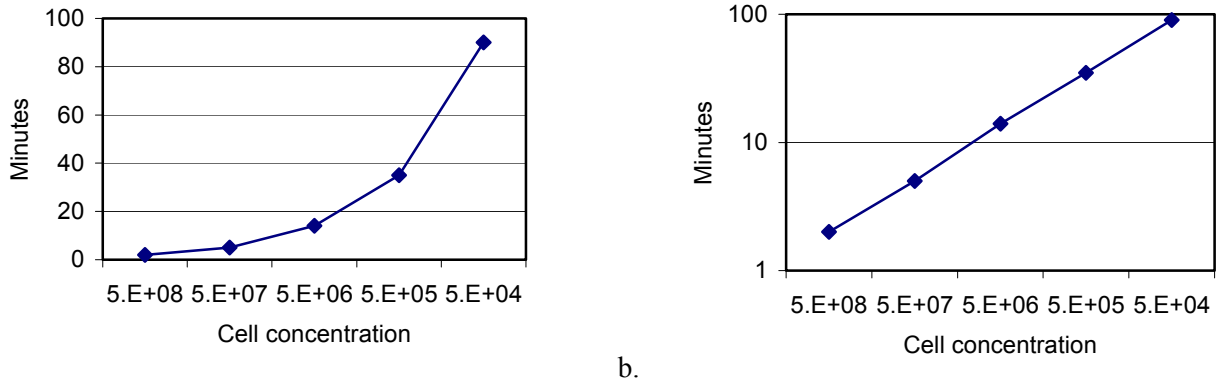


Figure 23. Responses of *B. globigii* spores in the DCF microplate viability assay. Spore were accelerated to germinated/outgrown viable cells at 37˚C for 3 hours, washed and assayed in the standard buffered-DCF assay format at 37˚C for 1 hour. Samples were run in quadruplicate with <3% coefficient of variation.

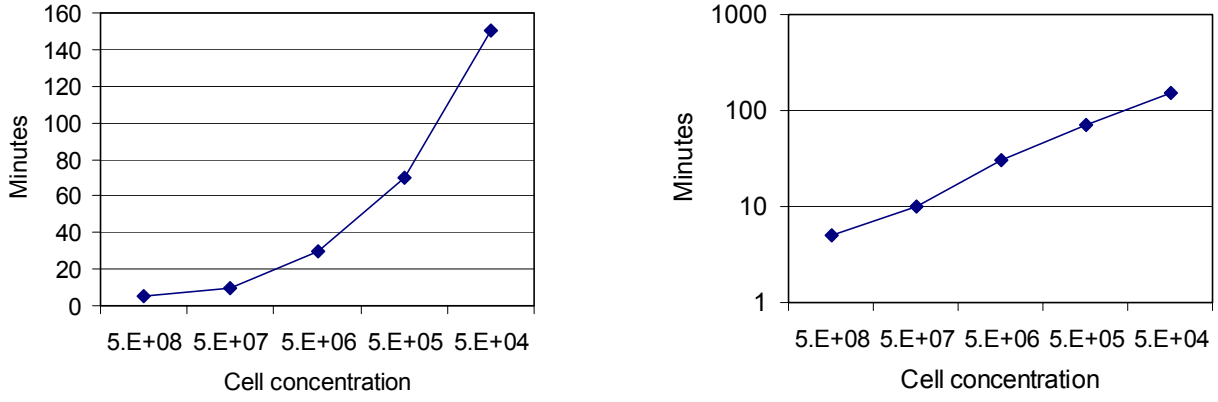
To determine the relationship between cell numbers and the fluorescence responses in the resazurin assay, the times for dilutions of accelerated spores to reach 50% of their maximal response were correlated with cell counts (CFU/ml). Based on the data generated in Figure 8, this time-course can be depicted as shown in Figure 24.



a. b.

Figure 24. Correlation between cell concentration and time to reach 50% maximum fluorescence signal response in the resazurin assay. Accelerated spores (37˚C, 5 hours) were washed and diluted into DCF reagent and fluorescence was measured every 5 minutes. a. linear time plot, b. logarithmic time plot.

In addition, we performed a similar set of time-course DCF experiments in the incubating, shaking 96-well microplate reader. Accelerated spores (5 hours at 37˚C) were washed 2 times with PBS and 20 µl of each dilution were added to 180 µl of DCF reagent. Then, 20 µl aliquots were serially transferred into 180 µl of the DCF reagent resulting in ten-fold dilutions to 10^{-5} (~5000 spore/ml). The reaction mixtures were incubated at 37˚C with fluorescence excitation at 485 nm and excitation at 535 nm measured every 5 minutes.



a. b.

Figure 25. Correlation between cell concentration and time to reach 50% maximum fluorescence signal response in the DCF assay. Accelerated spores (37˚C, 5 hours) were washed and diluted into DCF reagent and fluorescence was measured every 5 minutes. a. linear time plot, b. logarithmic time plot.

3.0 Summary and Discussion

The rationale for using rapid, fluorescent viability assays to monitor the efficacy of decontaminants on bacterial pathogens applies primarily to vegetative bacteria and not bacterial endospores. Unfortunately, in recent years the bacterial pathogen most relevant to biowarfare and bioterrorism is the spore-former *Bacillus anthracis*. The recalcitrant bacterial spore has no metabolic activities to measure in fluorescence-based viability assays. Thus before the efficacy of decontaminants on bacterial spores can be determined, the cryptic spores must be transformed into vegetative cells and enumerated using conventional methods. This can be done by first germinating the spores with inducers that initiate the process whereby spores lose their refractility and resistance to heat and chemicals. Heat-activated spores (e.g., 75˚C, 15 min) of *Bacillus* species rapidly germinate in the presence of L-alanine or inosine alone through binding of specific receptors in the spore and induction of dipicolinic acid (PDA) excretion and swelling. In contrast, non-activated spores cannot germinate well in either compound alone but rapidly germinate in the presence of both. In addition, germination of non-heat activated spores is an age-related phenomenon and old spore stocks often do not require any heat activation. Thus with appropriate accelerants (L-alanine and inosine), the syringe/filter viability assay format developed during this special contract can rapidly and effectively monitor the lethal effects of decontaminants for the most resistant of biological pathogens, the bacterial spore.

The early stages of the three month special contract "Viability Assays for Monitoring Decontamination of Pathogenic Bacteria" involved, i) growing, maintaining and storing spores of *Bacillus globigii*, ii) optimizing the swab-based collection and enumeration of spores from treated surfaces, iii) optimizing the acceleration of spores and outgrowth of viable cells and iv) integrating the accelerant and assay reagents into a syringe/filtration sampling and assay format. Swabbing methods for spore-treated surfaces employing Dacron swabs and glass slides were developed which had recovery rates of 80%-90%. Using a syringe/filter assembly comprised of a 5 ml polypropylene syringe and a glass microfiber membrane filter cartridge (13 mm, 0.45 μm dia) with the resazurin assay, we detected ~10^4 viable cells/ml which was derived from ~50 spores following acceleration at 37˚C for 7 hrs.

During the latter stages of the special contract, Luna adapted the syringe/filter resazurin assay format for use with swabbed samples from spore-treated surfaces to rapidly determine the lethality of the decontaminant DF-200 on spores of *B. globigii*. The DF-200 treated surfaces yielded no viable spores by the plate count method and accelerated broths from spore swabs had no response in the rapid resazurin assay. When used in the laboratory to rapidly screen formulations against simulated surface contaminations, the fluorescent viability assays can save considerable time and money while providing the quality data required for accurate assessment of a decontaminant's efficacy. When adapted for field use, these assay formats may eventually provide for simple, rapid means to monitor clean up efforts and facilitate redeployment of contaminated weapons and military gear.

In addition to developing the novel formats for assessing the effects of biocides on bacterial spores and spore-contaminated surfaces, we examined 96-well microtiter plate formats for use in cost-effective, rapid, "high-throughput" experiments. Using the Tecan GENios fluorescent microplate reader, viability dose-responses and time-course were performed with considerably less effort, time and cost than using cuvettes in a fixed holder fluorometer (e.g., a 96-well plate costs ~$1 whereas 96 microcuvettes cost ~$29). Using automated temperature control, microplate plate shaking and kinetic (time-based) fluorescence readings, we could assay all three tester strains against several concentrations of a decontaminant or permutations of reaction mixtures in a single microplate in 1-2 hours. Subsequently, future applications of the microplate viability assay formats could extend into other relevant fields. In the pharmaceutical area of antibiotic susceptibility testing, rapid and reliable means of determining minimal inhibitory concentrations (MIC) of antimicrobial agents toward common bacterial pathogens can be critical. In many cases, the delay in determining MICs by conventional broth or plate methods can make the difference between effective early therapy and prolonged or secondary infections. In the food industry, the fluorescence-based assays could be used to rapidly monitor milk and other liquid products for contamination by mixed microbial populations including the spore-forming bacilli.

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